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Neural progenitors from human embryonic stem cells

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The derivation of neural progenitor cells from human embryonic stem (ES) cells is of value both in the study of early human neurogenesis and in the creation of an unlimited source of donor cells for neural transplantation therapy. Here we report the generation of enriched and expandable preparations of proliferating neural progenitors from human ES cells. The neural progenitors could differentiate *in vitro* into the three neural lineages—astrocytes, oligodendrocytes, and mature neurons. When human neural progenitors were transplanted into the ventricles of newborn mouse brains, they incorporated in large numbers into the host brain parenchyma, demonstrated widespread distribution, and differentiated into progeny of the three neural lineages. The transplanted cells migrated along established brain migratory tracks in the host brain and differentiated in a region-specific manner, indicating that they could respond to local cues and participate in the processes of host brain development. Our observations set the stage for future developments that may allow the use of human ES cells for the treatment of neurological disorders.

ES cell lines are derived from the pluripotent cells of the early embryo[1-3]. ES cell lines can potentially maintain a normal karyotype infinitely on culture *in vitro* and can differentiate into any cell type[4]. ES cell lines have recently been derived from human blastocysts[5,6], and their potential to differentiate into neural lineages has been demonstrated both *in vivo* in teratomas, and *in vitro*[6-8]. The differentiation of human ES cells into neural progeny may serve as an *in vitro* model for the study of early human neurogenesis. Furthermore, it may enable the development of *in vitro* models of human neurodegenerative disorders, the creation of high-throughput screens for the discovery of neuroprotective and neurotoxic agents, and the identification of novel genes, growth and differentiation factors that have a role in neurogenesis. The potential use of human ES cells as a renewable source of neural cells for transplantation and gene therapy[9] also attracts much public attention.

When ES cells are induced to differentiate *in vitro*, they give rise to a mixture of progeny from the three embryonic germ layers[8,10]. However, we require a means to control differentiation of ES cells into a purified neural progenitor cell population to realize many of their potential applications in neuroscience and regenerative medicine in the central nervous system (CNS). In the mouse ES cell system, strategies for the generation of enriched preparations of proliferating neural progenitors have been developed[11,12]. The *in vitro*–generated neural progenitors could differentiate *in vitro* into both glial cells and functional postmitotic neurons[11]. Transplantation experiments have demonstrated the potential of mouse ES cell–derived neural progenitors to participate in brain development[13], to myelinate axons in host brain and spinal cord[14,15], and to promote recovery after spinal cord injury[16].

We have recently demonstrated that human ES cells can also give rise to neural progenitor cells *in vitro*, and have further demonstrated that the progenitors can differentiate *in vitro* into mature neurons[6]. Here, we extend this work, demonstrating the derivation of highly enriched and expandable populations of proliferating neural progenitors from human ES cells. Furthermore, the neural progenitors could differentiate *in vitro* into mature neurons, astrocytes, and oligodendrocytes. When grafted into the brain ventricles of newborn mouse, the human neural progenitors migrated into the host brain and differentiated in a region-specific manner, according to normal developmental cues, into progeny from the three fundamental neural lineages.

Results

Derivation and propagation of progenitor cells from human ES cells. To derive enriched preparations of neural progenitors, differentiation of human ES cells was induced by prolonged culture (three to four weeks) without replacing of the mouse embryonic fibroblast feeder layer[6]. One week after passage, changes in cell morphology could be identified mainly in the center of the colonies, indicating the initiation of early differentiation. At this time, the expression of transcripts of the neuroectodermal markers nestin and PAX-6 was demonstrated by RT-PCR (Fig. 1A). The expression of transcripts of neural markers could reflect either some constant background differentiation or the process of early neural differentiation.

During the next two weeks of culture, the process of differentiation was markedly accelerated, mainly in the center of the colonies, and cells with short processes that expressed the early neuroectodermal marker N-CAM (neural cell adhesion molecule) could be identified[6]. It appeared that the N-CAM$^+$ cells were growing out from adjacent but distinct areas that were composed of small, piled, tightly packed cells that did not react with the monoclonal antibody

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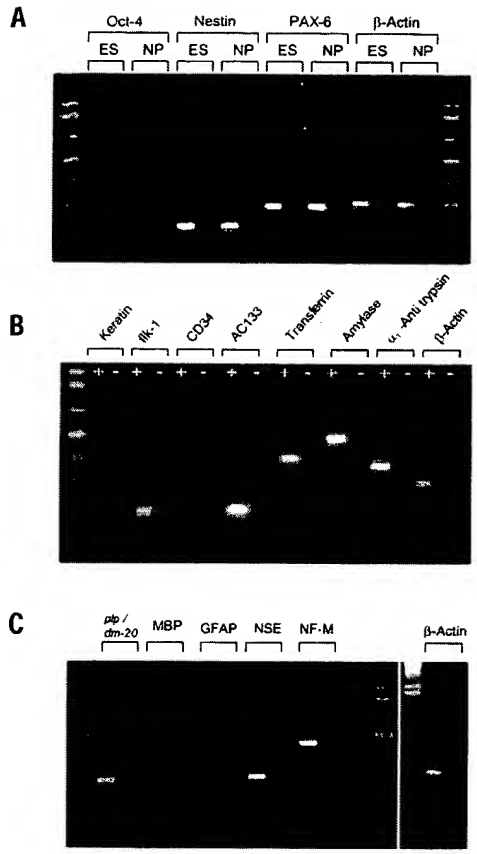

Figure 1. RT-PCR analysis of the expression of markers in human ES cell colonies, ES-derived spheres, and in differentiated cells originating from the spheres. (A) Oct-4, nestin, and PAX-6 in human ES cell colonies at one week after plating and in neural progenitor (NP) spheres. (B) The expression of non-neural marker genes in human ES cell–derived spheres. (C) Neuronal and glial markers in differentiated cells originating from human ES cell–derived neural progenitor spheres. All panels show 2% agarose gels stained with ethidium bromide. The symbols + and – indicate whether the PCR reaction was done with or without the addition of reverse transcriptase. A 1 kb plus DNA ladder was used in all panels. Oct-4 band is 320 bp, nestin 208 bp, PAX-6 274 bp, β-actin 291 bp, keratin 780 bp, Flk-1 199 bp, CD34 200 bp, AC133 200 bp, transferrin 367 bp, amylase 490 bp, α1-antitrypsin 360 bp, *plp* and *dm-20* are 354 bp and 249 bp, respectively, MBP is 379 bp, GFAP is 383 bp, NSE is 254 bp, and NF-M is 430 bp.

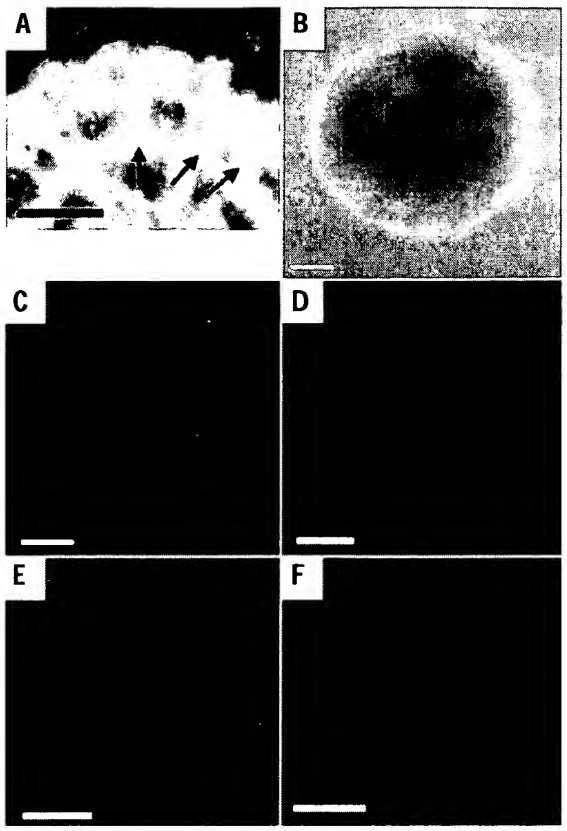

Figure 2. Analysis of morphology and marker expression in human ES-derived progenitor cells. (A) Dark-field stereomicroscopic photograph of a differentiating ES cell colony, four weeks after plating, with areas of cells (arrows) that are destined to give rise to neural progenitors. (B) Phase contrast micrograph of a sphere cultured in serum-free medium. (C–F) Indirect immunofluorescence staining of progenitor cells, 4–12 h after disaggregating of spheres and plating on adhesive substrate, for N-CAM, vimentin, nestin, and A2B5, respectively. Bars =1.6 mm (A), 100 μm (B), 25 μm (C, E, F), 35 μm (D).

GCTM-2, which identifies undifferentiated ES cells[6], and did not express the early neuroectodermal marker N-CAM (data not shown). These distinct areas had a uniformly white–gray and opaque appearance under dark-field stereomicroscopy (Fig. 2A), and could be identified in 54% of the colonies (67/124). They were surrounded by cells with diverse morphologies expressing a large array of somatic and extraembryonic markers, including muscle actin and desmin[6], α-fetoprotein, hepatocyte nuclear factor (HNF)-α, cardiac actin, and kallikrein (RT-PCR; not shown).

Assuming that the cells in the distinct areas gave rise to the adjacent N-CAM$^+$ cells, clumps of about 150 cells were mechanically isolated from these areas and replated in serum-free medium[17]. Under these culture conditions, the clumps formed free-floating spherical structures within 24 h.

Supplementing the medium with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), a growth factor combination that is known to be effective for the propagation of human fetal- and adult-derived neuroepithelial progenitors[17–20], facilitated sequential propagation and expansion of the sphere cultures. During the first two weeks in culture, some cell death was observed and the spheres gradually acquired a uniform round morphology (Fig. 2B). A detailed analysis of marker expression and the growth and differentiation potential of the cells within the spheres was conducted in three preparations that were separately derived and propagated.

The level of proliferation of the cells within the spheres was monitored indirectly by measuring the increase in the volume of the spheres over time. Most of the cells within the spheres were viable as demonstrated by Trypan Blue staining (94 ± 3.2%, $n = 47$ spheres). A positive correlation between the volume of the spheres and the number of cells within the spheres (Fig. 3B) was documented at various passage levels (5–15 weeks after derivation), indicating that an increment in sphere volume could be used as an indirect indication of cell proliferation. The spheres grew over an 18- to 22-week period, after which time the volume of the spheres was stable or declined. A relatively rapid growth rate was observed during the first five to six weeks after derivation, with a population doubling time of ~4.7 days. It was followed by a 10- to 16-week period of slow and stable cell growth with a population doubling time of ~2.5 weeks. This proliferative capability could potentially allow a significant expansion of the progenitor cell cultures (Fig. 3A).

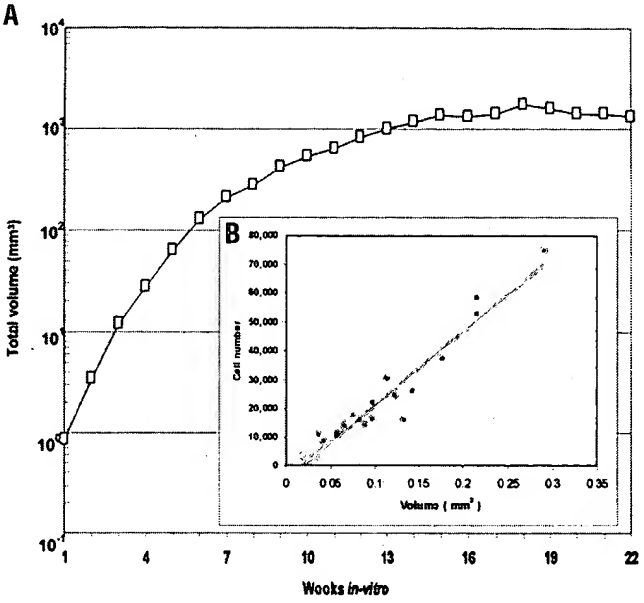

Figure 3. Cumulative growth curve for human ES-derived progenitor cells. (A) Continuous growth is evident during an 18- to 22-week period. The increment in the volume of the spheres was continuously monitored as an indirect measure of the increase in cell numbers. A linear positive correlation between the volume of the spheres and the number of cells within the spheres (B, insert) was maintained during cultivation.

Characterization of the progenitor cells within the spheres. Cells in the spheres expressed markers of neural progenitor cells, such as N-CAM (ref. 21; Fig. 2C), the intermediate-filament protein nestin[22] (immunostaining, Fig 2E; RT-PCR, Fig. 1A), A2B5 (ref. 23; Fig. 2F), vimentin[24] (Fig. 2D), and the transcription factor PAX-6 (Fig. 1A). The expression of these markers was maintained with prolonged cultivation *in vitro* (18 weeks).

To evaluate the proportion of neural progenitors in the cultures, spheres were disaggregated into single cells that were plated, fixed, and analyzed for the expression of the early neural markers (Fig. 2C–F). A high proportion of the cells expressed N-CAM (99 ± 1.6%, $n = 11$ experiments), nestin (97 ± 2.3%, $n = 10$ experiments), and A2B5 (90.5 ± 1.1%, $n = 6$). A lower proportion of cells were immunoreactive to the vimentin-specific antibody (67 ± 16.8%, $n = 9$ experiments). These proportions were stable during cultivation of the spheres (up to 18 weeks).

Oct-4 is a member of the POU-domain transcription factor family whose expression is limited in the mouse to pluripotent cells and is downregulated upon differentiation[25]. We have previously demonstrated a similar pattern of expression in human ES cells[6]. Oct-4 was not expressed by cells in the neural progenitor spheres, indicating that undifferentiated human ES cells were not present within the spheres (Fig. 1A).

To determine whether cells that had acquired markers of other tissues or lineages were present within the spheres, the expression of markers representing derivatives of mesoderm, endoderm, and epidermis were examined. Cells within the spheres expressed transcripts of markers of hematopoietic/endothelial progenitors (CD34, AC-133, Flk-1), endoderm (α1-antitrypsin, transferring, and amylase) and epidermis (keratin), as demonstrated by RT-PCR (Fig. 1B). Markers of extraembryonic endoderm were not expressed by the progenitors (α-fetoprotein and HNF-α, RT-PCR; not shown) or their differentiated progeny (low-molecular-weight cytokeratin and laminin immunostaining; not shown). The expression of transcripts of non-neural markers was evident after prolonged cultivation of the spheres. It could represent contamination by a small number of non-neural cells generated during the derivation of our cultures. Alternatively, it could represent plasticity of primitive neural progenitors that expressed markers, or gave rise to cells from other lineages[26,27]. Whatever the source, additional selection either on the basis of cell-surface markers[18] or on the expression of lineage-specific genes[12] may be needed to generate pure neural cultures.

***In vitro* neural differentiation.** The neural progenitors in the spheres could differentiate *in vitro* into derivatives of the three fundamental neural lineages. In general, differentiation was induced by plating whole spheres on an appropriate substrate in the absence of growth factors. Under these conditions the spheres attached rapidly, and cells migrated out to form a monolayer of differentiated cells (Fig. 4A).

For neuronal differentiation studies, spheres were plated on poly-D-lysine and laminin-coated dishes. After two to three weeks, cells that migrated out and formed a monolayer both displayed the morphology and also expressed the structural markers that are characteristic of immature neurons, such as β_{III}-tubulin (Fig. 4B), the 70 kDa neurofilament proteins (Fig. 4C), and neuron-specific enolase (NSE; Fig. 1C). Moreover, the differentiated cells expressed markers of mature neurons such as the 160 kDa neurofilament proteins (NF-M, Fig. 4D; RT-PCR, Fig. 1C), MAP-2ab (Fig. 4E), and synaptophysin (Fig. 4F). Furthermore, the cultures contained cells that synthesized glutamate, expressed glutamic acid decarboxylase (GAD; the rate-limiting enzyme in GABA biosynthesis), synthesized GABA and serotonin, and expressed tyrosine hydroxylase (TH; Fig. 4G–K). Neurons that synthesized GABA and glutamate were relatively abundant, comprising 35% and 15% of the neuronal population, respectively. TH- and serotonin-producing cells were relatively rare (<1%).

For glial differentiation studies, spheres were plated on poly-D-lysine- and fibronectin-coated dishes and were cultured first in the presence of EGF, bFGF, and platelet-derived growth factor-AA (PDGF-AA), followed by culture in the presence of tri-iodothyronine (T3). The combination of bFGF and PDGF-AA is known to promote the proliferation of glial precursor cells[14], whereas T3 has been shown to enhance differentiation of oligodendrocyte lineage cells from human embryonic neural spheres[28].

Differentiation into astrocytes was demonstrated by the presence of cells that expressed glial fibrillary acidic protein (GFAP) (Fig. 4L; RT-PCR, Fig. 1C). Oligodendrocyte lineage cells were infrequent under our culture conditions and few cells were immunoreactive to O4, an antibody recognizing oligodendrocyte-specific glycolipids[29] (Fig. 4M). Differentiation to the oligodendrocyte lineage was further confirmed by demonstrating the expression of RNA transcripts of both myelin basic protein (MBP) and the *plp* gene (Fig. 1C). The *plp* gene encodes the proteolipid protein and its alternatively spliced product DM-20, which are major proteins of brain myelin[21].

To evaluate the proportion of neurons versus glial cells following induction of differentiation, spheres that were propagated 10 weeks were disaggregated and plated on poly-D-lysine- and laminin-coated dishes and cultured in the absence of mitogens for five days. Fifty-seven percent of the cells were immunoreactive to anti-β_{III}-tubulin (a marker characteristic of immature neurons) and 26% to anti-GFAP. Therefore, at least 83% of the cells took on a neural fate. The potential of the neural progenitors to give rise to both neurons and glial cells *in vitro* was maintained for the duration of the 22 weeks of propagation.

Integration and differentiation in host brain. To explore the developmental potential of the human ES-derived neural progenitors *in vivo*, disaggregated bromodeoxyuridine (BrdU)-labeled

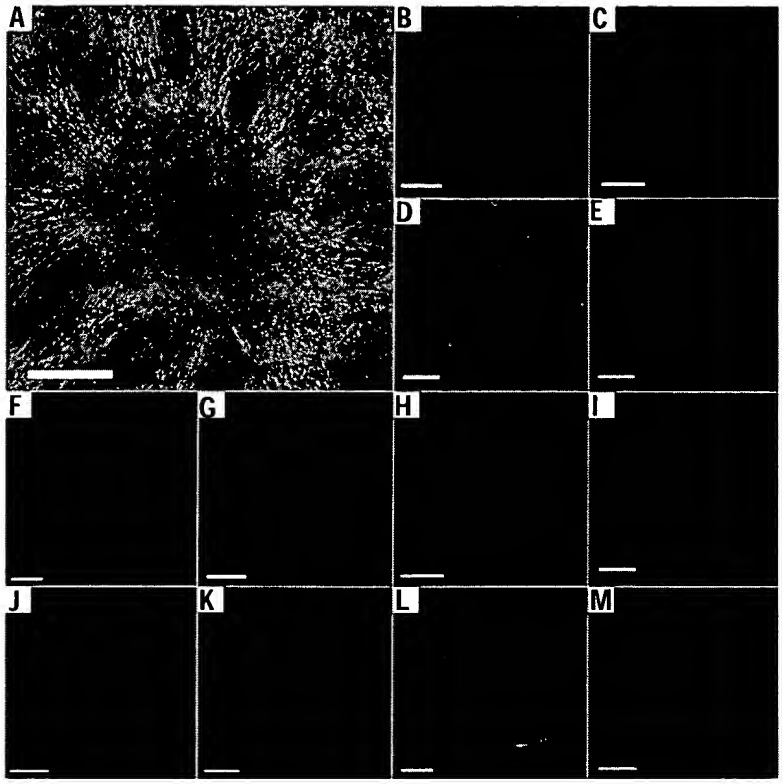

Figure 4. Phase contrast appearance and marker expression of differentiated cells originating from human ES-derived neural progenitor spheres. (A) Phase contrast micrograph of differentiated cells emanating from a sphere two weeks after plating onto an adhesive surface and culture in the absence of growth factors. (B–M) Indirect immunofluorescence microscopy of differentiated cells decorated with antibodies against the following neuronal and glial markers: β_{III}-tubulin (B), 70 kDa neurofilament proteins (C), 160 kDa neurofilament proteins (D), MAP2ab (E), synaptophysin (F), glutamic acid decarboxylase (G), GABA (H), glutamate (I), serotonin (J), tyrosine hydroxylase (K), GFAP (L), O4 (M). Bars = 200 μm (A, D), 50 μm (E), 20 μm (B, C, F–M).

spheres were implanted into the lateral cerebral ventricles of newborn mice[19]. Transplantation was performed 9–15 weeks after derivation of the neural spheres. Histological and immunochemical evaluation of serial brain sections was performed 4–6 weeks after transplantation. Numerous BrdU$^+$ cells were found in 9 out of 14 recipient animals, and successful engraftment was documented with donor cells from the three neural progenitor populations (Fig. 5). Transplantation efficiency was highly variable. BrdU$^+$ cells were not observed in the brain parenchyma of control animals that received transplantation of killed, BrdU-labeled, neural progenitors (Fig. 5D). The human origin of the cells decorated with anti-BrdU was confirmed by double labeling with a combination of anti-BrdU and anti-human specific ribonucleoprotein antibodies (Fig. 6A–D). The identity of the transplanted human cells was also confirmed by immunofluorescent staining with human-specific anti-mitochondrial antibodies (Fig. 6E–G). The distribution of cells that were immunoreactive with anti-BrdU and with human-specific anti-mitochondrial antibody in various regions of host brain was similar.

Brains that were examined a week after transplantation exhibited clusters of donor cells lining the ventricular wall (Fig. 5A). Four to six weeks following transplantation, human cells had left the ventricles and migrated in large numbers mainly as individual cells into the host brain parenchyma. The human cells demonstrated a widespread distribution in various regions of the host brain including periventricular areas, the entire length of the corpus callosum, fimbria, internal capsule, diencephalic tissue around the third ventricle, and dentate gyrus (Fig. 5B, E–H). Transplanted human cells also migrated anteriorily from the subventricular zone along the rostral migratory stream (Fig. 5C) and populated the olfactory bulb, indicating their potential to respond to local cues and migrate along established host brain tracts.

Differentiation *in vivo* into the three fundamental neural lineages was demonstrated by immunochemical studies using anti-human cell type–specific antibodies or double-labeling experiments with both anti-BrdU or anti-human specific ribonuclear protein (RNP) and anti-neural cell type–specific antibodies. Glial differentiation of the transplanted cells was abundant in the periventricular areas that consist of white-matter tracks where glial differentiation in the postnatal period is predominant. *In vivo* differentiation into astrocytes was demonstrated by immunochemical staining with anti-human-

Figure 5. Dissemination of transplanted BrdU$^+$ human ES-derived neural progenitor cells in the mouse host brain. (A) Two days after transplantation, most cells were found lining the ventricular wall. (B) After four to six weeks, most cells had left the ventricles (V) and populated the corpus callosum (CC), fimbria (fim), and internal capsule (ic). (C) Chains of BrdU$^+$ cells were found in the RMS. (D) In animals that were transplanted with dead BrdU-labeled cells, there was no BrdU staining in the brain parenchyma. (E) BrdU$^+$ cells in the periventricular white matter. (F) High magnification of BrdU$^+$ cells in the corpus callosum; in the cortical layer above the corpus callosum (*) there were no BrdU$^+$ cells. (G) High-magnification image showing BrdU$^+$ cells populating the fimbria. (H) Low-magnification image showing BrdU$^+$ cells in the dentate gyrus. Str, striatum; hipp, hippocampus.

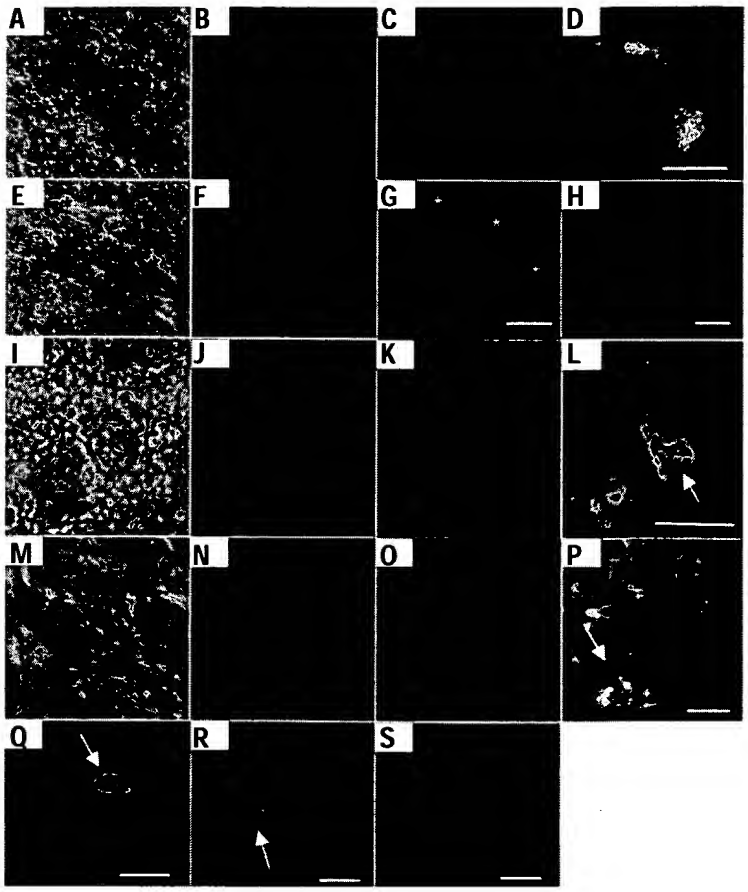

Figure 6. Identification of the transplanted cells in the brain by human and neural lineage–specific markers. (A–D) Nuclei (identified in Nomarski optics, panel A) were double labeled for BrdU (green fluorescence, panel B) and human-specific anti-RNP (red fluorescence, panel C). The nuclear co-localization (D) indicated that BrdU$^+$ cells were indeed of human origin. (E–G) A typical chain of transplanted cells in the corpus callosum, stained with human-specific anti-mitochondrial antibody. The mitochondrial staining (green fluorescence, panel F) on Nomarsky background (blue; panel G, cell nuclei indicated by asterisks) shows typical perinuclear localization. (H) A periventricular transplant-derived astrocyte detected by a human-specific anti-GFAP antibody. (I–L) A transplant-derived astrocyte from the periventricular region. The nucleus (identified by Nomarski optics, panel I) is labeled with BrdU (J, green fluorescence), indicating its origin from the graft and surrounded by GFAP staining (K, L). (M–P) A human oligodendrocyte progenitor cell identified in the periventricular region. The cell membrane (M, arrows) and nucleus (M, arrow and asterisk) are identified by Nomarski optics. Co-labeling of nucleus by anti-BrdU (N) and cell membrane by anti-NG-2 (O) are demonstrated in image (P). (Q) A CNPase$^+$ oligodendrocyte (green) in the corpus callosum, co-labeled with human-specific RNP (red). (R) A β_{III}-tubulin$^+$ neuron (green fluorescence) in the olfactory bulb, co-labeled with human-specific RNP (red). (S) Neuronal processes in the fimbria, stained with a human-specific anti-70 kDa neurofilament. Bars = 10 μm.

specific GFAP (Fig 6H) and by double labeling for BrdU and GFAP (Fig. 6I–L). Transplanted cells that differentiated into the oligodendroglial lineage were demonstrated by double immunostaining with anti-BrdU and anti NG-2 (a marker of oligodendrocyte progenitors[30]) or anti-human RNP and anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase; Fig. 6M–P and 6Q, respectively).

Neuronal differentiation of the human transplanted cells was specifically demonstrated in the olfactory bulb, a region where neuronogenesis occurs after birth (Fig. 6R). Neuronal processes of transplanted cells were also detected by a human-specific anti–light chain neurofilament antibody in the fimbria (Fig. 6S).

There was no histological evidence of teratoma or non-neural tissue formation in any of the recipient animals.

Discussion

Our findings show that a highly enriched population of proliferating neural progenitors may be derived from human ES cells. These neural progenitors are capable of extensive proliferation *in vitro* while retaining their potential to give rise to the three fundamental neural lineages and to participate in mammalian brain development. Derivation of proliferating, highly enriched tissue-specific progenitors from human ES cells, as exemplified here for the neural lineage, is expected to be highly valuable for the analysis of the stages of early development and for the development of a donor source for tissue reconstruction.

Our culture conditions promoted the undifferentiated proliferation of the human ES-derived neural progenitors for 20 weeks. Throughout cell propagation, expression of markers of early neuroectoderm was maintained, as was the progenitors' potential to differentiate into neurons and glial cells. The neural progenitors did not express markers of ES cells or the morphology and markers of differentiated neural cells. It should be noted that because the neural progenitors were not subjected to clonal analysis, it is not possible to determine whether our cultures contained multipotent human neural stem cells or a mixture of more restricted neural progenitors[31]. Nevertheless, our approach could distinguish among the early phases of human neurogenesis, including pluripotent ES stem cells, the proliferation of neural progenitors, and their differentiation into neurons and glial cells. This is the first step toward the development of more refined *in vitro* models that will distinguish between neural progenitors at various levels of commitment[26] and will allow the dissection of the cellular and molecular processes accompanying the various stages of development of the human nervous system.

Our data demonstrate that neural progenitors derived from human ES cells *in vitro* can respond appropriately to normal developmental cues *in vivo*. Following transplantation to the cerebral ventricles of newborn mice, the donor cells migrated in large numbers into the host brain parenchyma and became widely distributed. The engraftment efficiency was variable, and additional studies are required to determine to what extent, if at all, *in vivo* proliferation of transplanted cells contributed to the total number of human cells in host brains. Migration of the transplanted cells was not random, and the human progenitors followed established brain pathways, indicating that they respond to host's signals. The human ES cell–derived neural progenitors differentiated *in vivo* into neurons, astrocytes, and oligodendrocytes. Differentiation into neurons was demonstrated in the olfactory bulb where host differentiation into this lineage occurs in the postnatal period, whereas differentiation into astroglia and oligodendroglia was demonstrated in subcortical white-matter tracts where gliogenesis predominates and neurogenesis is complete[32]. These data demonstrate that cell fate was determined in a region-specific manner and according to the region's stage of development.

When ES cells, including those of human origin, are engrafted into various organs of young adult host mice, they may give rise to teratocarcinoma or teratomas[5,6]. We did not observe the formation of teratomas or non-neural tissues in any of the transplanted mice, and we also could not detect expression of the stem cell marker Oct-4 in the neural progenitor cultures. Thus, contamination by undifferentiated ES cells was probably eliminated by our selective derivation and propagation protocols. Nevertheless, given the expression of tran-

scripts of markers of non-neural lineages by the cells in our cultures, additional immunohistochemical and molecular studies are needed to determine whether non-neural human cells are generated in the host brain parenchyma. Thorough long-term studies are required to determine the safety of the transplantation of human ES cell–derived neural progeny, and to rule out potential hazards such as tumor formation or the development of cells from other lineages.

Data are accumulating rapidly regarding the signals and factors that govern the proliferation of neural progenitors and determine their fate during CNS development[31]. In this study we have used this knowledge to generate an enriched, expandable population of developmentally competent neural progenitors from human ES cells. This work serves as a platform for further manipulations with growth and differentiating factors that may eventually enable the derivation of specific neural cells[33], and may facilitate the use of human ES cells as a useful tool in basic neuroscience research and regenerative medicine.

Experimental protocol

Derivation and culture of progenitor cells. Human ES cells (HES-1 cell line[6]) with a stable normal (46XX) karyotype were cultured on mitomycin C mitotically inactivated mouse embryonic fibroblast feeder layer in gelatin-coated tissue culture dishes as described[6]. After three weeks of continuous culture, patches containing ~150 cells each were cut out from distinct areas within the differentiating ES colonies using the razor-sharp edge of a micro-glass pipette. Contamination by other cell types was avoided by paying careful attention to cut well within the distinct areas. The clusters of cells were transferred to plastic tissue culture dishes containing growth medium that consisted of Dulbecco's minimal essential medium (DMEM)/F12 (1:1), B27 supplementation (1:50), glutamine 2 mM, penicillin 50 units/ml, and streptomycin 50 μg/ml (Gibco, Gaithersburg, MD), and supplemented with 20 ng/ml human recombinant EGF and 20 ng/ml bFGF (R&D Systems, Inc., Minneapolis, MN). The clusters of cells developed into round spheres that were subcultured by dissection into quarters (by two no. 20 surgical blades; Swann-Morton, Sheffield, UK), every 7–21 days when their diameter exceeded 0.5 mm. Fifty percent of the medium was replaced every three to four days.

Analysis of growth. The increment in the volume of 24 spheres was monitored weekly starting from the first passage (one week after derivation). A stereomicroscope was used to measure the diameter of individual spheres, and their volume was calculated using the equation for the volume of a ball. The spheres were passaged every 7–21 days when the diameter of at least six spheres exceeded 0.5 mm. At each passage, six spheres (diameter >0.5 mm) were sectioned into quarters that were plated individually in a 24-well tissue culture dish. When growth was evaluated a week after passage, the sum of volumes of the daughter spheres was compared to the sum of volumes of the mother spheres.

Immunohistochemistry studies. Immunostaining of ES cell colonies to evaluate the expression of GCTM-2 and N-CAM was performed as described[6]. Standard protocols were used for the immunophenotyping of spheres, disaggregated progenitor cells, and differentiated cells. Fixation with 4% paraformaldehyde was used unless otherwise specified. Primary antibody localization was done by using swine anti-rabbit and goat anti-mouse immunoglobulins conjugated to fluorescein isothiocyanate (FITC 1:20; Dako, Carpinteria, CA), and goat anti-mouse IgM conjugated to Texas Red (1:50; Jackson Laboratories, West Grove, PA). Proper controls for primary and secondary antibodies revealed neither nonspecific staining nor antibody cross-reactivity.

To characterize the immunophenotype of cells within the aggregates, spheres that were cultivated 6–18 weeks were disaggregated and the single cells were plated on poly-D-lysine (30–70 kDa, 10 μg/ml; Sigma, St. Louis, MO) and laminin (4 μg/ml; Sigma), fixed after 4–12 h, and examined for the expression of N-CAM (acetone fixation, 1:10; Dako), nestin (rabbit antiserum, a gift of Dr. Ron McKay; 1:25), A2B5 (1:20; American Type Culture Collection, ATCC, Manassas, VA), and vimentin (methanol fixation, 1:20; Roche Diagnostics Australia, Castle Hill, NSW). Two hundred cells were scored within random fields (at 400×) for the expression of each of these markers, and the experiments were repeated at least three times.

For the study of the expression of extraembryonic endodermal markers, whole spheres were plated on poly-D-lysine and fibronectin (5 μg/ml; Sigma), cultured four weeks in growth medium without growth factors, and examined for the expression of low-molecular-weight (LMW) cytokeratin (Beckton Dickinson, San Jose, CA) and laminin (1:500; Sigma).

Neuronal differentiation was induced by culturing the spheres on poly-D-lysine and laminin in growth medium without supplementation of growth factors for two to three weeks. In some of the experiments, starting from the sixth day after plating, the medium was supplemented with all-*trans* retinoic acid (10^{-6} M; Sigma). Differentiated cells were analyzed for the expression of 160 kDa neurofilament protein (methanol fixation, 1:50; Chemicon, Temecula, CA), 70 kDa neurofilament protein (1:100; Chemicon), MAP2ab (1:100; Neomarkers, Union City, CA), glutamate (1:1,000; 1% (wt/vol) paraformaldehyde–1% (vol/vol) glutaraldehyde fixation; Sigma), synaptophysin (1:50; Dako), TH (Sigma), serotonin (1:1,000; Sigma), GAD (1:200, 1% (wt/vol) paraformaldehyde–1% (vol/vol) glutaraldehyde fixation; Chemicon; 1:200), GABA (1:1,000; Sigma), and β_{III}-tubulin (1:150; Sigma). To determine the proportion of neurons that synthesized the various neurotransmitters, at least 100 cells were scored within random fields of the outgrowth from differentiating spheres (at 400×) for the expression of β_{III}-tubulin and each of the neurotransmitters, and the experiments were repeated at least three times.

To enhance the differentiation toward the glial lineages, spheres were plated on poly-D-lysine and fibronectin, cultured two weeks in growth medium supplemented with recombinant human PDGF-AA (20 ng/ml), bFGF (20 ng/ml), and EGF (20 ng/ml), followed by two weeks in the presence of T3 (30 nM; Sigma) only. Differentiated cells were analyzed for the expression of GFAP (1:50; Dako) and O4 (1:10; Chemicon).

Reverse transcription (RT)-PCR analysis. Total RNA was collected from human ES cell colonies (one week after passage), from free-floating spheres, and from differentiated cells growing from spheres that were induced to differentiate to the neuronal or glial lineages as detailed above. Total RNA was isolated using RNA STAT-60 solution (TEL-TEST, Inc., Friendswood, TX) and was reverse-transcribed into complementary DNA (cDNA) with SuperScript First Strand Synthesis System (Gibco) using oligo (dT) as a primer according to the manufacturer's instructions. PCR was carried out using standard protocols with *Taq* DNA Polymerase (Gibco) or *Tfl* DNA Polymerase (Promega, Madison, WI). Primer sequences (forward and reverse) and the length of amplified products were as follows: Oct-4 (primers[34]); nestin, PAX-6, NSE, NF-M, *plp* (primers[35]); keratin, amylase, α1-antitrypsin (primers[8]); flk-1, CD34, AC133 (primers[36]); GFAP, MBP (primers[20]); *transferrin:* 5′-CTGACCTCACCTGGGACAAT-3′, 5′-CCATCAAGGCACAGC-3′ (367 bp); α-fetoprotein: 5′-CCATGTACATGAGCACTGTTG-3′, 5′-CTCCAATAACTCCTGGTATCC-3′ (338 bp); HNF-α: 5′-GAGTTTACAGGCTTGTGGCA-3′, 5′-GAGGGCAATTCCTGAGGATT-3′ (390 bp). As a control for messenger RNA (mRNA) quality, β-actin transcripts were assayed using the same RT-PCR and the following primers: 5′-TCACCACCACGGCCGAGCG-3′, 5′-TCTCCTTCTGCATCCTGTCG-3′ (291 bp). Amplification conditions were as follows: 94°C for 4 min followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, 72°C for 45 s, and extension at 72°C for 7 min. Products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

Transplantation to the developing brain. Spheres were cultured in the presence of BrdU (50 μM; Sigma) for 10 days. Fifty percent of the medium was replaced every three to four days with fresh medium containing fresh BrdU. The spheres were then disaggregated; 86% of the cells were viable as determined by Trypan Blue staining, and 40% were decorated by anti-BrdU. Approximately 50,000–100,000 cells (in 2 μl PBS) were injected into the lateral ventricles of newborn (P1) mice (Sabra mice; Harlan, Jerusalem, Israel) by using a glass micropipette (300 μm outer diameter) connected to a micro-injector (Narishigi Inc., Tokyo, Japan). Transplantation of dead, BrdU-labeled, human ES cell–derived neural progenitors served as control experiments. The neural progenitors underwent three cycles of freezing by plunging into liquid nitrogen and thawing in room temperature just before transplantation. At one to six weeks of age, recipients were anesthetized and perfused with 4% paraformaldehyde in PBS.

Detection and characterization of donor human neural progenitors *in vivo*. Serial 7-μm frozen sections were examined by immunostaining after postfixation with acetone or with 4% paraformaldehyde. The transplanted cells were detected by immunostaining with antibodies for BrdU

(1:20; Dako), anti-human specific RNP antibody (1:20; Chemicon), and anti-human specific mitochondrial antibody (1:20; Chemicon). BrdU antibody was detected by using the peroxidase-conjugated Vectastain kit (Vector Laboratories, Burlingame, CA), developed with diaminobenzidine (DAB), or by using goat anti-mouse IgG conjugated to Alexa 488 (1:100; Jackson). Anti-RNP and mitochondrial antibodies were detected with goat anti-mouse IgM conjugated to Cy5 and goat anti-mouse IgG conjugated to Alexa488, respectively (1:100; Jackson). Transplanted astrocytes were identified by double staining for BrdU and GFAP (1:100; Dako) or by anti-human specific GFAP (1:100; Sternberger Monoclonals Inc., Lutherville, MD). Anti-CNPase (1:100; Sigma) and anti NG2 (1:100; Chemicon) were used for the oligodendrocyte lineage. Neurons were detected by immunostaining with human-specific anti-neurofilament light chain (1:100; Chemicon) and anti-β_{III}-tubulin (antibody as detailed above; 1:100). Goat anti-rabbit conjugated to Cy5 (1:100; Jackson) and goat anti-mouse IgG conjugated to Alexa488 (1:100; Jackson) were used for detection of primary antibodies. Images were taken with a confocal microscope (Zeiss). All double-stain immunofluorescence signals were analyzed at multiple consecutive planes to ensure the co-localization of nuclear and cytoplasmic or membranal signals to the same cell.

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